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Expression, purification, crystallization and preliminary X-ray diffraction analysis of the apo form of $InsP_5$ 2-K from *Arabidopsis thaliana*

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Inositol 1,3,4,5,6-pentakisphosphate 2-kinase (IP_5 2-K) is a key enzyme that catalyzes the synthesis of phytic acid (IP_6) from inositol 1,3,4,5,6-pentakisphosphate (IP_5) and ATP. The first structure of IP_5 2-K, that from *Arabidopsis thaliana*, has been solved previously; it only crystallized in the presence of inositol, either the substrate IP_5 or the product IP_6, and failed to crystallize in its free state (without inositol). Based on structural analysis, a point mutation of IP_5 2-K (W129A) has been produced in order to overcome this limitation and obtain information about protein conformational changes upon substrate binding. Here, the production and crystallization of W129A IP_5 2-K in its free state and with bound nucleotide is described. These crystals differed from the native crystals and belonged to the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 66.00, b = 68.23, c = 105.80 Å and a = 63.06, b = 71.80, c = 100.23 Å, respectively. The crystals diffracted to resolutions of 2.22 Å (apo) and 2.05 Å (nucleotide bound) using synchrotron radiation and contained one molecule per asymmetric unit. The structures have been determined using the molecular-replacement method and refinement is being undertaken.

1. Introduction

Inositol polyphosphates (IPs) are a new class of signalling molecules that regulate multiple cellular activities, including chromatin remodelling and transcription, mRNA export, telomere-length regulation, RNA editing, exocytosis and translation (Michell, 2008). The levels of these compounds are regulated by different inositol phosphate kinases which phosphorylate the different positions of the inositol ring (Irvine & Schell, 2001).

One of the most prominent and well studied inositol polyphosphates is inositol 1,2,3,4,5,6-hexakisphosphate or phytic acid (IP_6). Many functions have been suggested for IP_6, including mRNA export and processing (Michell, 2008). IP_6 acts as a cofactor for different RNA-editing enzymes (Macbeth *et al.*, 2005) and is also required for the production of inositol pyrophosphates (Saiardi *et al.*, 2000), which are involved in apoptosis processes (Nagata *et al.*, 2005). In addition, phytate (the salt of phytic acid) is the major phosphate store in plant seeds; it can lead to malnutrition owing to the chelating power of IP_6 and to environmental phosphorus pollution (Raboy, 2003).

The enzyme in charge of IP_6 synthesis is inositol 1,3,4,5,6-pentakisphosphate 2-kinase (IP_5 2-K), which employs ATP to phosphorylate the 2-OH axial position of inositol 1,3,4,5,6-pentakisphosphate (Ives *et al.*, 2000). This enzyme is of key importance in mammals; its deletion causes early lethality in mice embryos (Verbsky *et al.*, 2005). The important role of this protein prompted us to crystallize and solve the first structure of an IP_5 2-K, that from *Arabidopsis thaliana*, in complexes with both substrates and products (Baños-Sanz *et al.*, 2010; González *et al.*, 2010). The protein crystallized in space group $P2_12_12_1$ with two molecules (unit-cell parameters a = 58.12, b = 113.59, c = 139.72 Å; PDB entries 2xal, 2xam, 2xan, 2xar and 2xao) or one molecule (unit-cell parameters a = 62.11, b = 65.66, c = 121.79 Å; PDB entry 4aqk) in the asymmetric unit.

Despite our efforts, crystals of the apo form (without inositol) were not obtained. Study of the apo form is crucial to provide a complete picture of the enzyme and the conformational changes that occur

upon binding of substrates and catalysis. An in-depth analysis of our previous structures suggested that several elements such as Trp129 could be involved in these changes owing to their role in maintaining a closed protein conformation and covering the active site. Therefore, we produced the W129A mutant of IP_5 2-K by site-directed mutagenesis. Here, we present the expression, purification, crystallization and preliminary X-ray analysis of this mutated IP_5 2-K. The new crystalline form obtained will be useful in shedding light onto the conformational changes that are undergone by this enzyme.

2. Experimental procedures

2.1. Protein expression and purification

The W129A mutant was produced by the site-directed mutagenesis technique using 5′-CGTCCGCTA**GCG**CGTGTTAATGC-3′ and 3′-GCATTAACA**CGC**GCTAGCGGACG-5′ as oligonucleotides and our previous wild-type construction as the template (Baños-Sanz *et al.*, 2010; González *et al.*, 2010) cloned in pKLSLt vector (Angulo *et al.*, 2011). The incorporation of the mutation was assessed by DNA sequencing. Transformation was performed in *Escherichia coli* Rosetta (DE3) pLysS strain; the culture was grown in 2TY medium and incubated with shaking until an OD_{600} of 1.0 was reached. Expression was induced with 0.3 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG) for 16 h at 289 K. The cell pellet was harvested at 3400*g* and 277 K and stored at 193 K until use.

Pellets were resuspended in buffer *A* (20 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 2 m*M* DTT) plus a Complete EDTA-free protease-inhibitor cocktail tablet (Roche) and disrupted with a French press. To eliminate cellular debris, the lysate was clarified at 34 500*g* for 45 min and filtered with 0.45 µ*M* syringe filters. The filtrated lysate was applied onto a Sepharose CL-6B column equilibrated in buffer *A* and the protein was eluted in buffer *A* plus 200 m*M* lactose. Protein-containing fractions were pooled and diluted threefold with 20 m*M* Tris–HCl pH 8.0 to reduce the salt concentration, loaded onto a heparin column, washed with buffer *B* (20 m*M* Tris–HCl pH 8, 50 m*M* NaCl, 2 m*M* DTT) and eluted with a gradient from buffer *B* to buffer *C* (20 m*M* Tris–HCl pH 8, 1000 m*M* NaCl, 2 m*M* DTT). The eluted fused protein was cleaved using TEV protease (protease:protein mass ratio 1:80), shaking the sample gently at 277 K overnight. IP_5 2-K, LSLt and TEV protease were separated by size-exclusion chromatography using a HiLoad 16/600 Superdex 200 column equilibrated in buffer *D* (20 m*M* Tris–HCl pH 8.0, 200 m*M* NaCl, 2 m*M* DTT). The sample produced an isolated peak that corresponded to an IP_5 2-K monomer. Pure protein fractions were pooled and loaded into a small Sepharose CL-6B column equilibrated in buffer *D* to remove residual fusion protein, if present. The final yield of the purification was 14.3 mg per litre of culture. The purity of the sample was confirmed by SDS–PAGE (Fig. 1). The W129A IP_5 2-K mutant was fully active (data not shown). The final pure sample was concentrated to 10 mg ml^{-1} and stored at 193 K.

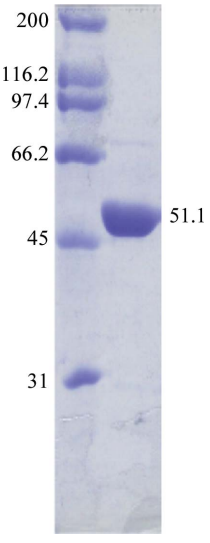

Figure 1
SDS–PAGE gel of purified W129A IP_5 2-K. Molecular weights are indicated in kDa.

2.2. Protein crystallization

Initial crystallization screening was performed by high-throughput techniques using a NanoDrop robot (Innovadyne Technologies Inc.) in 96-well plates (Innovaplate SD-2 microplates, Innovadyne Technologies Inc.). We tested six commercial screens (Index, Crystal Screen, Crystal Screen 2 and SaltRX from Hampton Research, JB Screen Classic 1–4 from Jena and PACT and JCSG-plus from Qiagen). The sitting-drop vapour-diffusion method was used, mixing 250 nl protein sample with 250 nl precipitant solution. Drops were equilibrated against a reservoir containing 60 µl screening solution. After two weeks, just one condition gave an initial hit: 0.2 *M* $LiSO_4$, 0.1 *M* Tris–HCl pH 8.5, 30%(*w*/*v*) PEG 4000 at 293 K (Fig. 2*a*). This condition was first refined by pH and PEG 4000 grid screening using the sitting-drop vapour-diffusion technique in 24-well Cryschem plates (Hampton Research). The drops (1 µl:1 µl) were equilibrated against a reservoir containing 0.5 ml crystallization condition. We obtained tiny blade-shaped crystals that were not suitable for diffraction studies in conditions consisting of 0.2 *M* $LiSO_4$, 0.1 *M* Tris–HCl pH 8.0, 32–34%(*w*/*v*) PEG 4000 (Fig. 2*b*). To improve the quality of the crystals, we performed streak-seeding from a seed stock generated from previously obtained crystals. The best crystals, which were suitable for X-ray diffraction, grew in 5 d at 293 K using 0.2 *M* $LiSO_4$, 0.1 *M* Tris–HCl pH 8, 29%(*w*/*v*) PEG 4000 (Fig. 2*c*).

To obtain nucleotide-bound crystals, we added 2 m*M* adenosine 5′-(*β*,*γ*-imido)triphosphate (AMPPNP; Sigma), a nonhydrolysable analogue of ATP, to the sample. We followed the same strategy for crystallization as was used for the unbound protein. Crystals were obtained using 0.5 *M* ammonium sulfate, 0.1 *M* sodium citrate tribasic dihydrate pH 5.6, 1.0 *M* $LiSO_4$. The optimization process was similar as before and a seeding procedure again yielded the best crystals, which were obtained using 0.5 *M* ammonium sulfate, 0.1 *M* sodium citrate tribasic dihydrate pH 5.6, 0.8 *M* $LiSO_4$.

2.3. Data collection and analysis

The W129A mutant crystals were cryoprotected by gradually increasing the PEG content from 29 to 35% before flash-cooling to 100 K in liquid nitrogen. A complete data set was collected from a W129A IP_5 2-K crystal at 100 K using an ADSC Q315r X-ray detector on the ID14-4 beamline at the European Synchrotron Radiation Facility (ESRF; Grenoble, France; Fig. 3). AMPPNP-bound crystals were cryoprotected using the crystallization solution plus 20% glycerol and flash-cooled to 100 K. Data for this sample were collected at 100 K using a MAR Mosaic 225 detector on the ID23-2 beamline at the ESRF.

Both data sets were processed using *iMOSFLM* (Battye *et al.*, 2011) and scaled with *SCALA* (Evans, 2006) from the *CCP*4 suite (Winn *et al.*, 2011). Data-collection and processing statistics are shown in Table 1.

3. Results and discussion

In order to capture a different conformation of IP_5 2-K by protein crystallography, we produced an IP_5 2-K point mutant (W129A) by site-directed mutagenesis. The W129A mutant expressed very well, yielding a similar amount of sample as the wild-type protein (Baños-Sanz *et al.*, 2010). However, W129A samples seemed to be more stable than the wild type, with less tendency towards aggregation upon concentration. Wild-type IP_5 2-K did not crystallize in the absence of inositol, but the introduction of the W129A mutation into the protein allowed us to obtain crystals of unbound and AMPPNP-bound forms.

The diffraction data indicated that the W129A mutant crystals belonged to space group $P2_12_12$, with unit-cell parameters a = 66.00, b = 68.23, c = 105.80 Å. The space group was the same for the AMPPNP-bound W129A mutant crystal, with unit-cell parameters a = 63.06, b = 71.80, c = 100.23 Å. The calculated values of the Matthews coefficient, 2.34 Å^3 Da^{-1} for W129A mutant crystals and 2.24 Å^3 Da^{-1} for AMPPNP-bound W129A mutant crystals, indicated the presence of one protein molecule per asymmetric unit, with a solvent content of 47.4 and 45.0%, respectively. The structure of the W129A mutant was solved by molecular replacement using *Phaser* (McCoy *et al.*, 2007) with our previous structure of IP_5 2-K bound to IP_5 and AMPPNP (PDB entry 2xan; González *et al.*, 2010) as a model.

Table 1
Data-collection and analysis statistics.

Values in parentheses are for the highest resolution shell.

	W129A IP_5 2-K	W129A IP_5 2-K–AMPPNP
Temperature (K)	100	100
Wavelength (Å)	0.9794	1.00
Source	ESRF	ESRF
Beamline	ID14-4	ID23-2
Space group	$P2_12_12$	$P2_12_12$
Unit-cell parameters (Å)	a = 66.00, b = 68.23, c = 105.80	a = 63.06, b = 71.80, c = 100.23
Resolution range (Å)	47.44–2.25 (2.37–2.25)	53.37–2.05 (2.16–2.05)
Unique reflections	169757 (24883)	207910 (30454)
Completeness (%)	99.6 (100)	100 (100)
Multiplicity	7.3 (7.4)	7.1 (7.3)
R_{merge}† (%)	7.1 (45.9)	9.8 (39.8)
Mean $I/\sigma(I)$	15.9 (4.2)	15.0 (4.9)
Matthews coefficient (Å^3 Da^{-1})	2.34	2.24
Solvent content (%)	47.41	45.03
No. of molecules in asymmetric unit	1	1
Wilson B factor (Å^2)	47.40	22.00

† $R_{merge}(I) = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observed amplitude of reflection hkl and $\langle I(hkl)\rangle$ is the mean amplitude for measurements of reflection hkl.

Our initial attempts to solve the structure using this model were unsuccessful; therefore, we defined two ensembles for the search

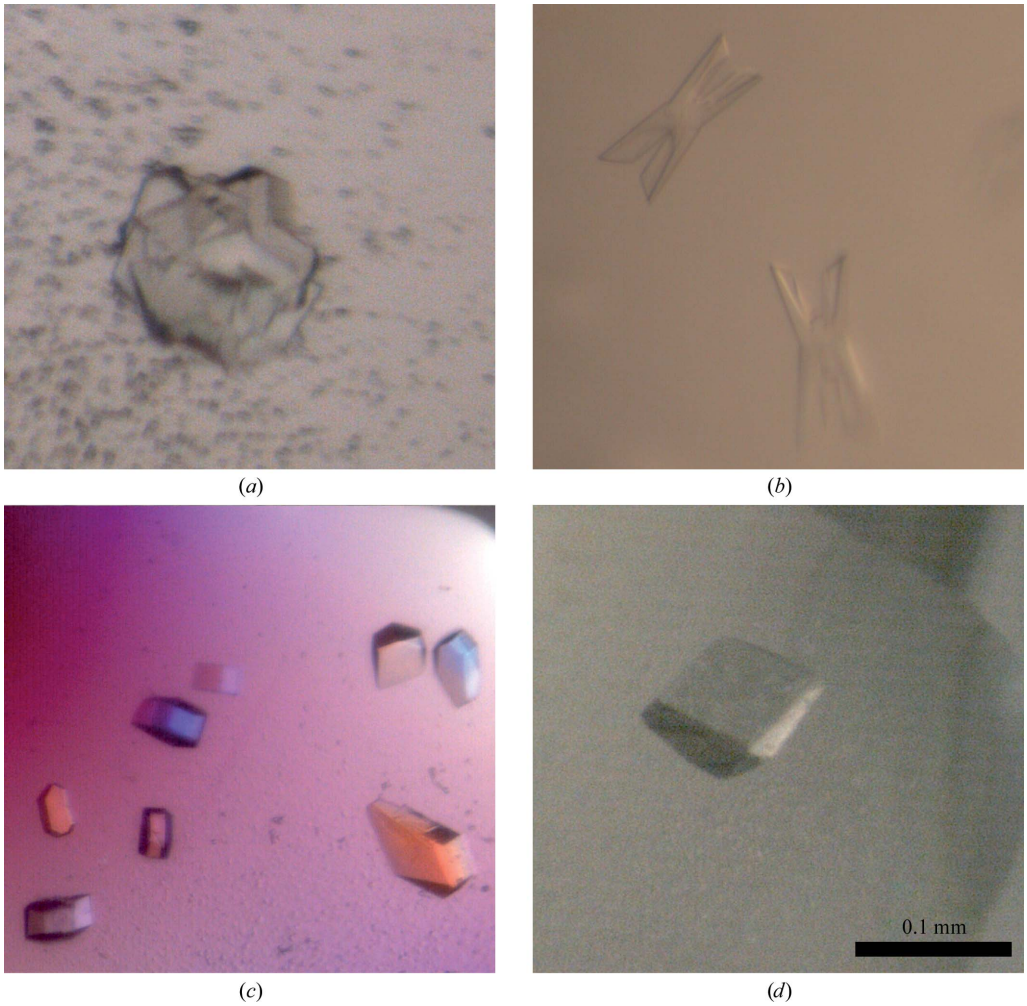

Figure 2
(*a*) Initial crystals of W129A IP_5 2-K. (*b*) W129A IP_5 2-K crystals obtained in refined conditions without seeding [0.2 *M* $LiSO_4$, 0.1 *M* Tris–HCl pH 8.0, 33%(*w*/*v*) PEG 4000]. (*c*) W129A IP_5 2-K crystals obtained after seeding [0.2 *M* $LiSO_4$, 0.1 *M* Tris–HCl pH 8.0, 29%(*w*/*v*) PEG 4000]. (*d*) AMPPNP-bound W129A IP_5 2-K crystals (0.5 *M* ammonium sulfate, 0.1 *M* sodium citrate tribasic dihydrate pH 5.6, 0.8 *M* $LiSO_4$).

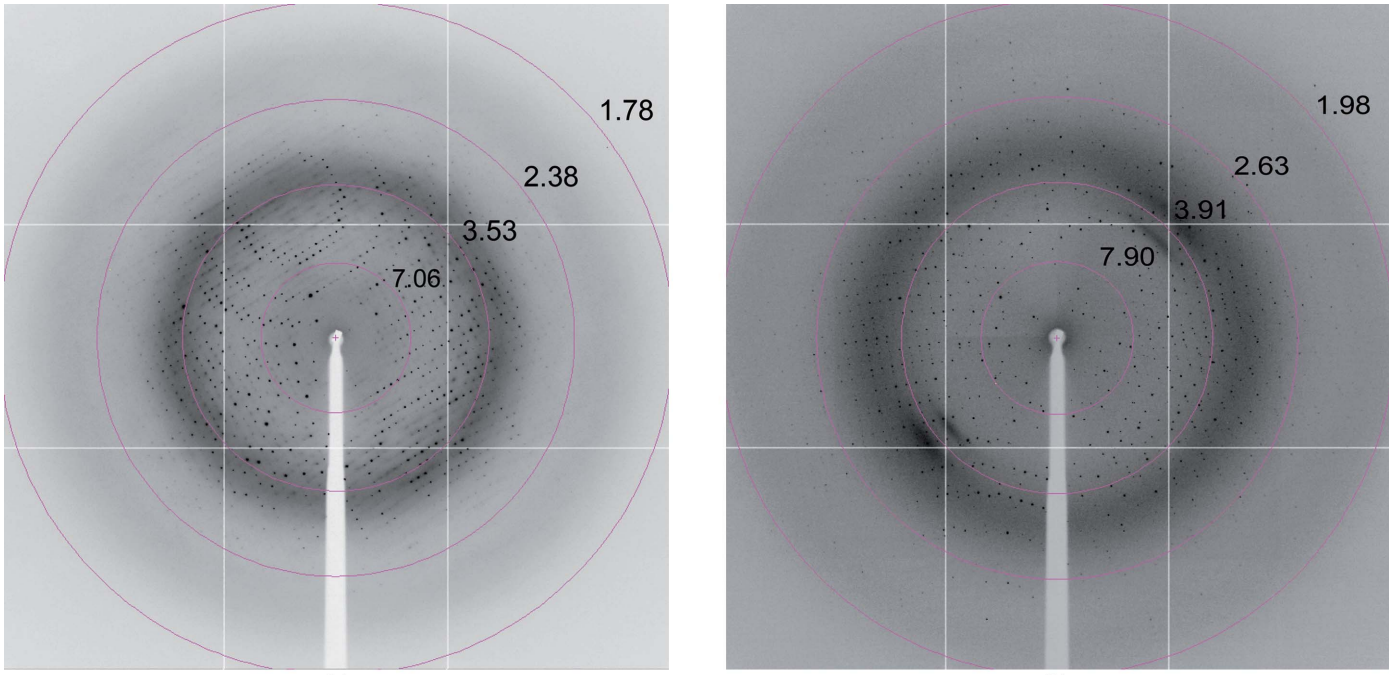

Figure 3
X-ray diffraction patterns for (*a*) apo-form W129A IP_5 2-K crystals and (*b*) AMPPNP-bound W129A IP_5 2-K crystals obtained using synchrotron radiation. The resolution of the rings (in Å) is labelled.

consisting of the N-terminal domain (residues 3–150) and the C-terminal domain (residues 160–437). Using this approach, we found a unique solution with a log-likelihood gain (LLG) of 1456.47 and a translation-function *Z* score (TFZ) of 23.4. The same approach was carried out to solve the AMPPNP-bound structure. The necessity of separating the protein into two domains to obtain a good solution probably indicates the existence of a conformational change between the inositide-bound form and the unbound form, as expected.

This is a good example of how a simple mutation can alter the crystallization behaviour of a protein. In this case, Trp129 is involved in both crystal packing and active-site closure and its mutation has probably altered both features. Structural refinement is in progress and will allow this hypothesis to be checked and the conformational changes of IP_5 2-K upon substrate binding to be characterized.

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